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by Estrogen

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Michael M. Sanders 9/28/99

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## INTRODUCTION (Adapted from 97/98 report)

The overall goal of our research has been to investigate the regulation of eucaryotic gene expression by steroid hormones. Through the use of differential display, we discovered that two mRNAs are rapidly repressed by estrogen in chick oviduct. Sequence analysis demonstrated that one of these shares 73% identity at the amino acid level with the human multidrug resistance-associated protein (hMRP). This protein is a member of the ATP-binding cassette (ABC) transmembrane transporter family. As members of this family are implicated in the development of resistance to therapeutic drugs, the ability to regulate the amount of MRP by estrogen selectively in mammary cells may provide a novel means of enhancing the efficacy of chemical interventions for breast cancer. Thus, the hypothesis under investigation is that estrogen directly represses the transcription of the human *MRP1* gene. The original goals of these studies were to determine whether clone #44 is a chick homologue of one of the human *MRP* genes, whether clone #41 represents another yet unidentified member of the multidrug resistance transporter family, whether expression of the human *MRP* homologue is repressed by estrogen, and whether that repression represents an effect on transcription or mRNA stability. Our broader hypothesis is that at least some estrogen-independent breast cancer cells become resistant to chemotherapeutic drugs when estrogen receptor is no longer capable of repressing expression of the *MRP1* gene. These proposed studies will provide the foundation upon which to design experiments that are directly applicable to designing innovative therapies for breast cancer based on manipulating the expression of the *MRP1* gene.

## BODY (Adapted from 97/98 report)

### TECHNICAL OBJECTIVES:

***Specific Aim I:*** To characterize clones #44 and #41 to determine whether they are homologues of the human *MRP* gene family

***Specific Aim II:*** To determine whether the human *MRP1* gene is regulated by estrogen

***Specific Aim III:*** To determine whether the regulation by estrogen is at the level of transcription, and, if so, to begin to define the critical regulatory mechanisms

## **RATIONALE, METHODS, AND RESULTS:**

***Specific Aim I: To characterize clones #44 and #41 to determine whether they are homologues of the human MRP gene family***

### **Rationale:**

Preliminary results suggested that two members of the *MRP* transporter family have been cloned from chick oviduct. Clone #44 appeared to be the orthologue of human *MRP1*. Based on the tissues in which it is expressed, clone #41 did not appear to be human cMOAT (*MRP2*), the only other *MRP* family member cloned at that time (1). The goal of this Specific Aim was to more completely characterize the chick cDNAs as the basis for extending the observation regarding the regulation by estrogen to the human. As both chick clones were less than 300 bp, the first objective was to obtain longer cDNAs for each so that sequence comparisons could be made.

### **Task 1: Cloning and sequencing of a larger cDNA for clone #44:**

As described in the 97/98 report, this task is complete and a manuscript describing those results is in review (Appendix A). This is essentially the same manuscript that was submitted as in preparation with the 97/98 report. However, we delayed submission of that paper until we could include the results of experiments proposed in Specific Aim III (see below).

### **Task 2: Cloning and sequencing of the cDNA for clone #41:**

This technical objective continues to be problematic. In the last annual report, we indicated that a new estrogen-withdrawn chick oviduct cDNA library was under construction to use for screening. However, we failed to get a sufficient number of independent clones in that library to warrant screening as most of the bacteriophage (phage) did not have cDNA inserts upon subsequent analysis. Therefore, rather than risk additional delay, we commissioned Clontech to make the library from poly(A)<sup>+</sup> RNA that we supplied. Although Clontech agreed that the RNA that we provided was intact and of sufficient quality, after two months they also were unable to produce an acceptable cDNA library. Therefore, we repeated the process by supplying them with more poly(A)<sup>+</sup> RNA. Again, several weeks passed before they admitted defeat and conceded that they were unable to make the library for unknown reasons. Thus after about a 6 month delay, we still had no suitable cDNA library to use for screening. We next turned again to making the library ourselves using a Clontech kit modified to use small amounts of RNA (SMART PCR cDNA Library Kit) and apparently suitable for cloning rare mRNAs. As of now, we have a phage library that appears to contain greater than 650,000 independent clones before amplification based on blue-white screening. We are in the process of excising clones in the form of plasmids. Thus, it is our expectation that we are now in a position to continue this task. We hope that clones will be obtained in the near future that include at least part of the coding sequence so that we can

determine whether this is a novel or previously identified gene and whether it is part of the ABC transporter family. As indicated in the previous report, we have already undertaken considerable characterization of the regulation of the gene (Appendix B from 97/98 report, Figs. 1 and 3 - 6), so once the identity is established, a manuscript can be submitted promptly.

**Specific Aim II: To determine whether the human *MRP* gene is regulated by estrogen**

**Rationale:**

As chick MRP mRNA rapidly decreases ( $t_{1/2} = \sim 45$  min) upon injection of estrogen (Appendix A, Fig. 4), this raises the intriguing possibility that the human gene is also regulated by estrogen. This contention is supported by the observation that human MRP mRNA is markedly reduced in estrogen-responsive tissues such as ovary, brain, and liver (2). Additional circumstantial evidence comes from the observations that the human breast cancer MCF-7 cell line typically loses functional estrogen receptors as the cells acquire resistance to drugs (3, 4). The ultimate goal of this specific aim is to determine whether the human *MRP1* gene is regulated by estrogen. If it is, this may explain how at least some estrogen-independent breast cancers become resistant to chemotherapeutic drugs. Because of the difficulties associated with determining whether the *MRP1* gene is regulated by estrogen in humans, these studies will use human breast cancer cell lines, particularly MCF-7 cells.

**Task 3: Treatment of MCF-7 cells with estrogen agonists and antagonists:**

Significant progress has been made on this task since during the last year. In particular the quantitative, competitive reverse transcription PCR (RT-PCR) assay using an internal control molecule (5) has been established. This initially met with some difficulty as it was necessary to test a number of primer pairs before finding a set that would specifically amplify human MRP mRNA and generate an internal control molecule (ICM) to yield only single bands on a gel. Two primers have been designed (#460 and #461) that are homologous to human MRP sequences 4061 to 4082 and 4569 to 4597, respectively, that work well in this assay. Similarly, primers to synthesize the ICM have been designed (#460 and #546, bases 4596 to 4568 and 4493 to 4459) such that the ICM is 103 base pairs shorter than the product from the wild type target product. The assay conditions have been worked out and are now linear and quantitative (See Appendix B, Figure 1).

Once the assay was established, experiments were done to address the question of whether estrogen represses the amount of MRP mRNA in MCF-7 cells. MCF-7 cells were passaged without estrogen for several passages and then  $1 \times 10^{-7}$  M  $17\beta$ -estradiol was added to replicate dishes for 0, 0.5, 1, 2, or 4 hours. RNA was isolated using the S.N.A.P. Total RNA Isolation Kit from Invitrogen. Reverse transcription reactions were performed essentially as described in the cDNA Cycle Kit (Invitrogen). Results of one such experiment

are depicted in Appendix B, Figures 2 and 3. The data indicate that MRP mRNA is rapidly degraded in response to estrogen treatment with a half-life of 2.5 hours. This is somewhat slower than was seen in the oviduct cells, where the half-life is closer to 45 minutes. The basis for this discrepancy is unclear but may be due to 1) different assay methods- QC RT-PCR versus northern blot, 2) different species- human versus chick, or 3) different molecular mechanisms. Nonetheless, estrogen does have a profound effect on the levels of MRP mRNA in human cells as well as in chick. At this time, experiments are in progress to test other estrogen agonists and antagonists as proposed in the grant, but no results are available as yet.

#### **Task 4: Treatment of MCF-7 cell line derivatives with estrogen agonists and antagonists:**

The goal of these studies is to determine the levels of *mrp* gene expression in cell lines that express estrogen receptor but are resistant to estrogen antagonists. These experiments have not yet been initiated because we have thus far been unable to obtain the appropriate cell lines. Although we have requested the LY-2 (6), MCF7/MIII (7), and MCF7/LCCI (7) cells, all of which are ER positive but antiestrogen resistant, the investigators have not responded. We are currently in the process of contacting them to ascertain whether we can obtain all or some of these lines. In addition, another line (MCF7/LCC2) is resistant to TAM but not to the steroidal antiestrogens ICI 182,780 or ICI 164,384 (7), and we want to acquire that one as well. Other cell lines with comparable characteristics are also being sought. By comparing the expression of the *MRP1* gene in these and other cell lines that vary in their responsiveness to estrogen agonists and antagonists and in their resistance to drugs, it should be possible to address the question of whether some breast cancers become resistant to mixed estrogen antagonists like TAM because they induce rather than repress expression.

#### **Specific Aim III: To determine whether the regulation by estrogen is at the level of transcription, and, if so, to begin to define the critical regulatory mechanisms**

##### **Rationale:**

The rapid loss of MRP mRNA in the chick oviduct after treatment with estrogen could reflect an effect on the transcription of the gene, on the stability of the resultant mRNA or both. Although no one has examined the half-life of MRP mRNA, that for *mdr* (multidrug resistance) mRNA is 30 - 60 minutes (8), which is consistent with the half-life of MRP mRNA observed in chick. The goal of this task is to determine the molecular basis for the rapid loss of MRP mRNA after treatment with estrogen.



**Task 5: Do nuclear run-on assays with nuclei from MCF-7 cells.**

To determine whether estrogen is repressing the transcription rate of the *mrp* gene, nuclear run-on assays were done using nuclei isolated from chick oviduct that had been withdrawn or treated with estrogen. The nuclear run-on assays were performed as we have done before (9 - 11). This experiment has been done three times using the complete cDNA clone as the probe, and no signal was seen from the *MRP* cDNA slots despite the fact that signals were seen with two transcription factors,  $\delta$ EF1 and HNF3 $\beta$  (data not shown and 10, 11). This indicates that the level of transcription of the *mrp* gene is very low, even without estrogen, further suggesting that the level of regulation by estrogen is post-transcriptional. The implication is that the level of regulation by estrogen is post-transcriptional, probably at the level of mRNA stability.

To approach this question in another way, oviduct cell cultures were treated with and without estrogen and with and without actinomycin D, an inhibitor of RNA polymerase. At various times after actinomycin D treatment, the cells were harvested, and the RNA was collected and measured using the QC RT-PCR assay. Unfortunately, after several months trying this experiment, we decided that the oviduct primary cell culture system was not suitable for this experiment. The problem is that the cells are refractory to estrogen for about eight hours after isolation from the chicks as most of the estrogen receptors, as determined by measuring estrogen receptor mRNA, are lost (data not shown). We attempted to manipulate the cells after this refractory period, but if we wait that long to add estrogen, the cells never regain estrogen responsiveness. In other words, unless the cells are cultured from the beginning with estrogen, they do not recover their ability to respond. Another complication was that *MRP* mRNA levels drop during the cell isolation so that it was necessary to keep the cells without estrogen for a while in culture before the *MRP* levels could increase. As a result of these technical difficulties and the inordinate amount of effort spent trying to overcome them, we have decided to abandon this approach and pursue what we expect to be a more tractable system, the MCF-7 cells. A manuscript based on the work in chick has been submitted and will suggest, although not prove, that the effects of estrogen are post-transcriptional. Thus, this task is considered complete.

**Task 6: Do pulse chase experiments with MCF-7 cells.**

Now that we have established that estrogen does repress human *MRP* mRNA levels, then the MCF-7 cell line can be used to address the question of whether estrogen is acting at the transcriptional or post-transcriptional level. MCF-7 cells will be cultured initially without estrogen in charcoal-stripped media. At zero time estrogen will be added and the cells will be harvested at 2 and at 4 hours. The nuclei will be isolated and subjected to nuclear run-on analysis. If the signal is too low, as was the case with the oviduct cells, then we will perform the actinomycin D experiment as described in the grant and summarized above. We fully expect that we will have the results of this experiment in a relatively short time.

**Task 7: Create an MRPCAT reporter vector and transfect into MCF-7 cells.**

From the minimal transcriptional activity observed from the *MRP* gene in oviduct cells, it seems unlikely that estrogen is acting at the level of transcription. However, the results from Task 6 will verify or refute that conclusion. Therefore, this task is on hold until Task 6 is completed.

**Task 8: Make and transfect additional MRPCAT reporter constructs:**

The results from Task 7 are required before proceeding with this Task.

**Task 9: Investigate DNA-protein binding interactions.**

The results from Task 6 are required before proceeding with this Task.

**Task 10: Define the sequences in MRP mRNA that are regulated by estrogen.**

Although the results are not completely solid at this point, it seems likely that estrogen modulates the stability of MRP mRNA. Therefore, it is appropriate to begin generating the constructs required for this Task.

## REVISED STATEMENT OF WORK

While the work on this project has proceeded slower than projected due to technical problems and to the fact that the transcription rate of the *MRP* gene is extremely low, significant progress has been made. Nonetheless, the time frame has shifted such that all of the proposed experiments will not be completed prior to the termination of the grant (8/30/00). However, it is my intention to continue the project using other resources until all of the Specific Aims are completed. The revised projected time frame is outlined below.

### **Task 1: Cloning and sequencing of a larger cDNA for clone #44:**

Completed

### **Task 2: Cloning and sequencing of the cDNA for clone #41:**

Months 25 - 29

### **Task 3: Treatment of MCF-7 cells with estrogen agonists and antagonists:**

Months 25 - 30

### **Task 4: Treatment of MCF-7 cell line derivatives with estrogen agonists and antagonists:**

Months 29 - 35

### **Task 5: Do nuclear run-on assays with nuclei from MCF-7 cells.**

Months 26 - 27

### **Task 6: Do pulse chase experiments with MCF-7 cells.**

Months 28 - 32

### **Task 7: Create an MRPCAT reporter vector and transfect into MCF-7 cells.**

I anticipate that this task will become irrelevant. However, if the data indicate otherwise, this task will take months 32 - 38

### **Task 8: Make and transfect additional MRPCAT reporter constructs:**

This task may be irrelevant. If it is relevant, then it will take months 38 - 48

**Task 9: Investigate DNA-protein binding interactions.**

This task may be irrelevant. If it is relevant, then it will take months 40 - 52

**Task 10: Define the sequences in MRP mRNA that are regulated by estrogen.**

Months 32 – 50

### **KEY RESEARCH ACCOMPLISHMENTS**

- Preparation of a cDNA library from withdrawn chick oviduct for cloning out more clone #41
- Development of a quantitative, competitive RT-PCR assay for human MRP mRNA
- Demonstration that human MRP mRNA is repressed by estrogen
- Determination of the half life of human MRP mRNA in response to estrogen
- Demonstration that the transcription rate of the human MRP gene is extremely low and not detectable by nuclear run-on assays

### **REPORTABLE OUTCOMES**

- Manuscript in review (Appendix A)
- Partial clone of chick MRP cDNA
- Faculty position obtained by post-doctoral student (Diane Dean) who has been working on this project for the past year based, in part, on her research plan to continue working on this project for the long-term

### **CONCLUSIONS**

Although the research project has not progressed as rapidly as planned, the observation that human MRP mRNA is down-regulated by estrogen with a half life of about 2.5 hours is a key and seminal observation. This result takes on new significance with the recent observations that MRP mRNA is present in 70% of breast cancer tissues, that its level of expression is significantly elevated in cancerous versus noncancerous breast tissue, and that it is much higher in patients that relapse ten years after chemotherapy (12, 13). Furthermore, this increase is specific for MRP as MDR gene expression is not increased in primary breast cancer. Thus, one of the major hypotheses underlying the proposed research has been verified, albeit by other groups. Thus, these observations are highly significant as they demonstrate for the first time that MRP may be useful as a marker for poor prognosis in patients with breast cancer. Furthermore, our observations raise the possibility that one or more of the selective estrogen receptor modulators (SERMs) may be useful in treating those resistant cancers.

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## APPENDIX A

### Down-Regulation of the Chick Multidrug Resistance-Associated Protein (*MRP*) Gene by Estrogen

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Running Title: Estrogen regulates the multidrug resistance-associated protein  
gene



**Abstract**

Although a number of genes have been identified whose transcriptional activities are up-regulated by estrogen, relatively few have been discovered that are down-regulated. However, gene repression is a common mode of signal transduction by the nuclear receptors. In hopes of determining whether estrogen can directly down-regulate genes, attempts were made to identify genes that are direct targets of the estrogen receptor and whose activities are down-regulated by it. Because the development and differentiation of the chick oviduct is dependent upon estrogen, this seemed an appropriate model system for testing this hypothesis. mRNA was isolated from estrogen-treated and estrogen-withdrawn chick oviduct and was subjected to differential display polymerase chain reaction. Surprisingly, one of the products that was down-regulated by estrogen encoded the chick homologue of the human and mouse multidrug resistance-associated protein (*MRP*) gene. The partial chick *MRP* (*chMRP*) cDNA clone contains 1322 base pairs and shows 73% identity with the human cDNA clone. Translation of the *chMRP* cDNA clone shows 73% amino acid identity with both the human and mouse proteins. The regulation of the *chMRP* gene by estrogen was assessed by northern blot analysis. In the oviduct, estrogen rapidly attenuates the amount of *chMRP* mRNA. A 50% decrease occurs within thirty minutes of estrogen administration and a 70% decrease by 1 hour, which is comparable to the level observed with chronic repression by estrogen. *chMRP* mRNA is present in many other tissues including the heart, lung, brain, kidney, skeletal muscle, and intestine but is undetectable in the liver. This study indicates that in estrogen-responsive tissues such as chick oviduct, the regulation of *chMRP* gene expression is

controlled by estrogen.

## Introduction

Multidrug resistance (MDR) occurs in many different tumor types and presents an obstacle to the treatment of these tumors (1). The MDR phenotype is characterized by the resistance of the tumor to chemotherapeutic agents that are structurally and mechanistically unrelated. MDR can occur inherently, as in renal and colon carcinomas, or can be acquired during treatment of the tumor by chemotherapeutic agents as in lymphomas and breast carcinomas (1). The MDR phenotype is derived at least in part to the expression of two genes, *MDR1* which encodes P-glycoprotein and *MRP* which encodes the multidrug resistance-associated protein (MRP) (2 - 5). Both P-glycoprotein and MRP belong to the ATP-binding cassette (ABC) transmembrane transporter family and are characterized by multiple membrane spanning domains and nucleotide binding domains (6). It is thought that MDR is conferred upon tumor cells by P-glycoprotein or MRP through the transport of chemotherapeutic agents out of a cell by way of these transmembrane transporters.

The human *MRP* gene was cloned and characterized from the small-cell lung cancer cell line H69AR and was shown to provide multidrug resistance to transfected cells (4, 5). The amino acid sequence of MRP indicates that it belongs to the superfamily of ABC transmembrane transporter proteins. Members of the ABC transmembrane transporter superfamily are ubiquitous from bacteria to man and transport a variety of molecules including amino acids, sugars, inorganic acids, polysaccharides, peptides, and proteins (6). While the physiological role of MRP remains largely unknown, it can act as an ATP-dependent transporter of cysteinyl leukotrienes, glutathione disulfide, and steroid conjugates including 17 $\beta$ -estradiol 17-( $\beta$ -D glucuronide) (7, 8).

Estrogen plays a critical role in the differentiation and development of the chick oviduct. The action of estrogen in regulating oviduct differentiation occurs through nuclear estrogen receptors, which regulate either the increase or decrease in transcription of specific genes (9, 10). To further understand the mechanism of estrogen-regulated gene expression in the chick oviduct, identification of primary targets of the estrogen receptor is necessary. Using differential display PCR (DD-PCR) (11, 12), a fragment of an estrogen down-regulated gene showing homology to the human *MRP* gene was cloned. Investigation into the regulation of chick *MRP* gene expression by estrogen is presented in this report. Understanding the mechanism of estrogen action in regulating *MRP* expression could prove useful in the elucidation of the function of MRP in development and differentiation and possibly provide clues as to its regulation in the treatment of tumors.

## **Materials and Methods**

**Animals.** Sexually immature female white leghorn chicks were subcutaneously implanted with two 10 mg diethylstilbestrol (DES), a synthetic estrogen, pellets (Hormone Pellet Press, Leawood, Kansas) for 2 weeks. "Stimulated" chicks retained the DES pellets while the pellets were removed from "withdrawn" chicks 5 days prior to use. For acute estrogen stimulation, withdrawn chicks were injected in the wing vein with 25 mg/kg 17 $\beta$ -estradiol (dissolved in propylene glycol). The chicks were then sacrificed at 0.5, 1, 2, and 4 hours after injection.

**Differential display polymerase chain reaction (DD-PCR) and**

**cloning of the chMRP cDNA.** Oviducts from estrogen-stimulated and -withdrawn chicks were collected and total RNA was isolated using an RNeasy RNA Isolation Kit (Qiagen). DD-PCR was carried out as previously described (11, 12) with modifications. Reverse transcription was carried out using oligo dT<sub>11</sub>G, dT<sub>11</sub>C, and dT<sub>11</sub>A primers in separate reactions. The PCR was carried out using the original oligo dT<sub>11</sub>N primer and a single random primer (5'-TGACGTACAC-3'). cDNAs were compared between the DES-stimulated and -withdrawn RNA samples on a 6% denaturing polyacrylamide sequencing gel. Each time point was done with at least Replicate chicks were used as a source for each time point. Differentially displayed cDNA fragments were excised from the sequencing gel and reamplified using the same primers used in the original PCR reaction. The amplified cDNAs were cloned into a T-tailed pBluescript vector. The Gene Trapper Kit (LifeTechnologies) was used to obtain a larger cDNA fragment of the chMRP gene from a cDNA library constructed from withdrawn chick oviduct mRNA using a cDNA library kit (LifeTechnologies). The chick withdrawn oviduct cDNA library was screened using the Gene Trapper kit with an oligonucleotide primer (5'-CTGGCCACCCCTATAGCTGC-3') designed from the differential display cDNA clone. cDNA clones were sequenced on an ABI DNA sequencer in the Institute of Human Genetics Microchemical Facility at the University of Minnesota.

**RNA isolation and northern blot hybridization.** Oviducts were removed from chicks, and the RNA was isolated using RNAzol (TelTest). Total RNA (20µg/lane) was electrophoresed in a 0.8% agarose/formaldehyde gel (20 mM MOPS, 1 mM EDTA, 5 mM sodium acetate, pH 7.0, and 2.2 M formaldehyde)

and transferred to Nytran membrane (Schleicher and Schuell) by capillary transfer. The blots were UV crosslinked using a Stratalinker (Stratagene) and prehybridized at 42°C for a minimum of 3 hours in 50% formamide, 6X SSC, 0.5% SDS, 5X Denhardt's and 100 µg/ml salmon testes DNA. Random labeled (Stratagene) cDNA probes labeled with  $^{32}\text{P}$ -dCTP were added to the prehybridization solution, and the blots were hybridized for 16 hours at 42°C in a rotating hybridization oven. The blots were washed at room temperature for 15 minutes in 2X SSC and 0.1% SDS, followed by 15 minutes at 42°C in 2X SSC and 0.1% SDS, and then for 15 minutes at 42°C in 0.2X SSC and 0.1% SDS, and then put on film. The cDNA probe for *chMRP* used in northern blot analysis was the 266 bp DD-PCR product isolated by cutting the *chMRP*-BS clone with either *EcoR* I or *Rsa* I followed by gel isolating the DNA fragment using a Qiagen gel isolation kit. 18S RNA probes were generated from a mouse 18S RNA clone obtained from American Type Tissue Collection.

## Results

**Partial cloning and sequence of the chick multidrug resistance-associated protein (*chMRP*) gene.** In an effort to understand the regulation of genes involved in chick oviduct development, differential display (DD) was used to clone genes that are down-regulated by estrogen. Sexually immature chicks were implanted with DES, a synthetic estrogen, pellets for at least two weeks prior to DD analysis to initiate oviduct growth and development. After the two weeks of initial DES stimulation, the DES pellets were removed for five days from the chicks that are designated as "withdrawn" (W/D). The chicks

in which the DES pellets were not removed are designated as "stimulated" (Stim). DD was performed using total RNA isolate from withdrawn and stimulated chick oviducts using an oligo dT<sub>11</sub>N primer and a single random primer. cDNA bands generated by the DD-PCR technique were compared between the estrogen-withdrawn and -stimulated RNA samples. cDNAs that appeared to be down-regulated by estrogen were isolated, re-amplified, subcloned, and sequenced. Sequence analysis of a 266 base pair DD-PCR clone that was differentially expressed in withdrawn and stimulated oviducts showed 73% identity at the nucleotide level with the human multidrug resistance-associated protein (*huMRP*) gene. To further confirm that the DD-PCR clone was in fact the chick homologue of *huMRP*, a larger 1322 bp clone (Figure 1) was obtained by screening an estrogen-withdrawn chick oviduct cDNA library. Nucleotide alignment of the *chMRP* cDNA clone and the *huMRP* gene shows 73% identity over the 1322 bp of the *chMRP* clone. Alignment of the translated *chMRP* clone with the human and murine MRP protein indicates a 73% identity and 79% similarity at the amino acid level (Figure 2). The similarity at both the nucleotide and amino acid level between the *chMRP* cDNA clone and the human and mouse *MRP* genes indicates that the clone is the chicken homologue of the human MRP gene and is part of the superfamily of ABC transmembrane transporters.

**Estrogen regulation of *chMRP*** To confirm that *chMRP* is indeed differentially expressed in estrogen-stimulated and estrogen-withdrawn chicks, northern blot analysis was carried out using total RNA isolated from chick oviduct (Figure 3). RNA isolated from withdrawn chick oviduct shows strong

hybridization of the chMRP DD-PCR probe to an mRNA species of approximately 6.6 kb. In contrast, the stimulated oviduct lane shows a dramatic decrease in the hybridization signal relative to the withdrawn lane.

To determine whether estrogen regulates chMRP mRNA acutely, withdrawn chicks were injected with 17 $\beta$ -estradiol and, at the indicated times, the oviducts were removed and RNA isolated. As shown by northern blot analysis (Figure 4A and 4B), chMRP mRNA is abundant in the estrogen-withdrawn oviduct (time 0 hour) but rapidly decreases by thirty minutes after injection of estrogen. Densitometric analysis of the northern blots indicates that the chMRP mRNA levels decrease by 70% within one hour, to a level equal to that is observed with chronic estrogen administration (Figure 4B). These results indicate that the *chMRP* gene is down-regulated in chick oviduct and that the decrease in chMRP mRNA occurs acutely, within one hour of estrogen administration.

**Tissue distribution of chMRP** To determine whether chMRP is present in other tissues in the chick, northern blot analysis was used to determine chMRP levels in RNA isolated from estrogen-withdrawn oviduct, liver, brain, heart, kidney, lung, skeletal muscle, and intestine (Figure 5A and 5B). The results demonstrate that chMRP mRNA is most abundant in oviduct, heart, and lung but is also expressed, albeit at about 75% lower levels, in brain, skeletal muscle, kidney, and intestine. chMRP mRNA was not detectable on our northern blots in liver. These results are comparable to those found with murine MRP, which is abundant in lung, heart, kidney, testes, and muscle and is also expressed in very low levels in the liver (13).



## Discussion

Using the technique of differential display, a partial clone was isolated corresponding to the chick multidrug resistance associated protein gene, *chMRP* (Figure 1). Sequence analysis indicates that the *chMRP* cDNA clone is a homologue of the previously cloned human and murine *MRP* genes (Figure 2) (4, 13). Over the length of the 1322 bp *chMRP* clone, there is significant homology at both the nucleotide and amino acid level with the human and murine *MRP* genes. While the human and murine *MRP* genes are 88% identical at the amino acid level, the *chMRP* clone encodes a peptide that is 79% identical to human and murine *MRP* (13). Structurally, *MRP* belongs to the family of ATP-binding cassette transmembrane transporter proteins. The members of this family consist of integral membrane ATP-binding proteins that transport a variety of molecules (6). The region of the *chMRP* gene that was cloned is located between the two nucleotide binding domains. Although members of the ABC transmembrane transporter superfamily share structural and functional similarity, identity at the primary sequence level is low and most of the sequence similarity resides in the nucleotide binding domains. Within the ABC transmembrane transporter superfamily, *MRP* is most closely related to the *YCF1* gene (43% amino acid identity) (14) but also has sequence identity with the cystic fibrosis transmembrane regulator (19%) (15), the rat  $\beta$  cell sulfonylurea receptor (29%)(16), and the *ltgpa* gene (32%) (17, 18). The high degree of identity between the *chMRP* clone and the human and murine *MRP* genes indicates that it is indeed the chicken *MRP* homologue rather than a new member of the ABC transporter superfamily.

These studies also indicate that the *chMRP* gene is acutely down-regulated by estrogen in the chick oviduct. Estrogen is critical for the development and differentiation of the chick oviduct and is involved in the activation of gene expression in cells that express estrogen receptor. However, control of gene expression and oviduct development may also include the down-regulation of genes by estrogen. As shown in Figure 4, an early response to the administration of estrogen is the down-regulation of the amount of *chMRP* mRNA. Regulation of other members of the ABC transmembrane transporter family by steroids has been shown. Mouse MDR mRNA is increased by estrogen and progesterone in uterine epithelial cells (19, 20), and expression of the cystic fibrosis transmembrane regulator gene is also increased by estrogen in uterine epithelial cells (21, 22). However, our results are the first to show down-regulation of a member of the ABC transmembrane transporter family by steroids. The rapid decrease in *chMRP* mRNA observed may be due to the decrease in gene transcription or to the decreased stability of the mRNA. Interestingly, in uterine epithelial cells estrogen increased the stability of the MDR mRNA (19). This indicates that estrogen has an effect on both the transcription rate and stability of the mouse MDR mRNA. Although estrogen may decrease the rate of transcription of the *chMRP* gene in chick oviduct, the extremely rapid decrease in *chMRP* mRNA suggests that there is also an estrogen-induced decrease in its stability as well.

The promoters for the *MRP* gene (23, 24) and the *mdr* gene (25 - 28) were cloned. Both the *MRP* and *mdr1* genes lack distinctive TATA box elements and have multiple sites of transcriptional initiation. The transcription factor Sp1 is involved in the regulation of both genes (23, 29). However, all the regulatory

elements necessary or important for expression of *MRP* have yet to be determined. Since the oviduct is one of the major estrogen-responsive tissues in the chick, another being the liver, it is probable that in the chicken *MRP* is regulated by estrogen in a tissue-specific manner.

Our results indicate that the *chMRP* gene is rapidly down-regulated by estrogen in the chick oviduct. While there is evidence from *in vitro* studies that *MRP* can confer the MDR phenotype, it is not clear what role *MRP* plays in the MDR phenotype in tumors (5). Determination of the elements required for activation or repression of *MRP* would be beneficial in understanding and perhaps treating tumors that exhibit the multidrug resistant phenotype, especially those that are responsive to estrogen..

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## Figure Legends

### FIGURE 1

**Partial cloning of the chicken multidrug resistance-associated protein gene.** Differential display-PCR was used to clone a fragment of the *chMRP* gene that is differentially expressed between estrogen-stimulated chicks and -withdrawn chicks. Additional sequence of the *chMRP* gene was cloned using the GeneTrapper kit (LifeTechnologies). A BLAST search indicated that the 1332 bp of the *chMRP* gene that was cloned aligns with the human MRP sequence.

### FIGURE 2

**Alignment of chMRP amino acid sequence with human and murine MRP.** The 1332 bp of the *chMRP* clone were translated and aligned with the human MRP (humMRP) and murine MRP (murMRP) proteins. The human and murine MRP amino acid sequences are 73% identical to the chMRP sequence. Numbering corresponds to the cloned chMRP fragment. The huMRP and murMRP sequences are from amino acids 832 to 1277 and 832 to 1272, respectively.

### FIGURE 3

**ChMRP mRNA is down-regulated by estrogen in the chick oviduct.** Northern blot analysis was carried out using 20 µg total RNA isolated from estrogen-withdrawn (W/D) and estrogen-stimulated (STIM) chick oviduct. The 266 bp *chMRP* differential display cDNA clone was used as the probe. The

lower panel shows the 28s and 18s rRNA bands stained with EtBr to indicate RNA loading. The chMRP probe hybridizes to a 6.6 kb band that is more abundant in the estrogen-withdrawn oviduct lane than the estrogen-stimulated oviduct lane.

#### FIGURE 4

**chMRP mRNA expression is acutely down-regulated in the oviduct by estrogen.** To determine whether the estrogen-induced decrease in chMRP mRNA occurs acutely, chicks that had the DES pellets withdrawn for five days were injected intravenously with 25 mg/kg 17- $\beta$ -estradiol. Oviducts were harvested at the indicated times (0, 0.5, 1, 2, and 4 hours) and total RNA isolated. A.) Northern blot analysis was carried out using the *chMRP* clone as a probe and an 18s rRNA probe was used as a correction for sample loading. B.) A plot of the chMRP/18s rRNA values indicates that chMRP mRNA decreases rapidly after the injection of 17- $\beta$ -estradiol. chMRP mRNA levels decrease by 50% within 30 minutes and by 70% within one hour, which is comparable to levels observed with chronic estrogen administration.

#### FIGURE 5

***chMRP* gene expression varies between tissues.** Tissue distribution of chMRP mRNA was determined by northern blot analysis using tissues from chicks withdrawn from estrogen for five days. A.) The northern blot was probed with the *chMRP* clone and an 18s rRNA probe. B.) The chMRP mRNA/18s rRNA values were determined by densitometry. chMRP mRNA is most abundantly expressed in the chick oviduct, heart, and lung. The brain, skeletal muscle,

kidney, and intestine show lower levels of chMRP mRNA expression. chMRP mRNA was not detected in the liver.



chMRP .....CGCGGC 6  
 humMRP GAAGAACAAGACGCGGATCTTGGTCACGCACAGCATGAGCTACTTGCCGC 2500  
 chMRP CGCGTCGACCAATTCTGGTAATGACTGATGGAGAAATCTCTGAGATGGGC 56  
 humMRP AGGTGGACGTCATCATCGTCATGAGTGGCGGCAAGATCTCTGAGATGGGC 2550  
 chMRP TCCTACCAGGAGCTGCTGAAGCAGGATGGAGCTTTTGCAGAGTTCCCTTCG 106  
 humMRP TCCTACCAGGAGCTGCTGGCTCGAGACGGCGCTTCGCTGAGTTCCCTGCG 2600  
 chMRP TACGTACGCTAATGCTGAACA...AAGCATGGAGAGCAGTGATGCAAGT 152  
 humMRP TACCTATGCCAGCACAGAGCAGGAGCAGGATGCAGAGGAGAACGGGGTCA 2650  
 chMRP AGTCCAT.....CTGGAAAGGAACGAAAGCCCGTAGAAAATGGAGTC 194  
 humMRP CGGGCGTCAGCGGTCCAGGGAAGGAAGCAAAGCAAATGGAGAATGGCATG 2700  
 chMRP CTTGTGAATGACGCCCCCTGGAAAGCTGATGCATCGGCAGCTCAGTAACTC 244  
 humMRP CTGGTGACGGACAGTGCAGGGAAGCAACTGCAGAGACAGCTCAGCAGCTC 2750  
 chMRP CTCCACATACAGCAGAGAACTGGGAAGTCACAGCACCAGAGCAGCACAG 294  
 humMRP CTCTCTCTATAGTGGGGACATCAGCA.....GGCACCACAACAGCACCG 2794  
 chMRP CAGAGCTGCAGAAGCCTCTTGCAGAGA...AGAATTCCTGGAACTGACA 341  
 humMRP CAGAACTGCAGAAAGCTGAGGCCAAGAAGGAGGAGACCTGGAAGCTGATG 2844  
 chMRP GAGGCTGACACAGCAGAGACTGGGAGGGTAAAGGCAACAGTATACTGGGA 391  
 humMRP GAGGCTGACAAGGCGCAGACAGGGCAGGTCAAGCTTTCCGTGTACTGGGA 2894  
 chMRP ATACATGAAAGCTATTGGACTCTATATCTCTTTTCTGAGCGTTTTCCTCT 441  
 humMRP CTACATGAAGGCCATCGGACTCTTCATCTCCTTCCTCAGCATCTTCTTT 2944  
 chMRP TTATGTGTAACCATATAGCCTCCCTGGCTTCCAACACTACTGGCTAAGTTTA 491  
 humMRP TCATGTGTAACCATGTGTCCGCGCTGGCTTCCAACACTATTGGCTCAGCCTC 2994  
 chMRP TGGACAGATGATCCGGTTGTCAATGGGACACAGCAGTACACAAATGTCAG 541  
 humMRP TGGACTGATGACCCCATCGTCAACGGGACTCAGGAGCACACGAAAGTCCG 3044  
 chMRP ACTGGGAGTATATGGAGCGCTGGGAATTTCTCAAGGTATTGCTGTGTTTG 591  
 humMRP GCTGAGCGTCTATGGAGCCCTGGGCAATTCACAAGGGATCGCCGTGTTTG 3094  
 chMRP GCTACTCGATGGCTGTGTCAATAGGAGGAATATTGCTTCACGGCACCTG 641  
 humMRP GCTACTCCATGGCCGTGTCCATCGGGGGGATCTTGGCTTCCCGCTGTCTG 3144

FIGURE 1

chMRP CACCTCGACCTGCTGCACAATGTTCTCAGGTCTCCAATGAGTTTCTTTGA 691  
 ||| | ||||| ||||| | || ||||| ||||| ||||| |||||  
 humMRP CACGTGGACCTGCTGCACAGCATCCTGCGGTACCCATGAGCTTCTTTGA 3194  
 chMRP ACGTACACCCAGTGGAAATTTAGTGAACCGTTTCTCTAAGGAGATAGATA 741  
 || | ||||| || | ||||| ||||| ||||| ||||| | ||  
 humMRP GCGGACCCCACTGGGAACCTGGTGAACCGCTTCTCCAAGGAGCTGGACA 3244  
 chMRP CCATTGACTCTACCATTCACCAATCATCAAGATGTTTCATGGGCTCAACA 791  
 | | |||| | || | ||||| ||||| ||||| |||||  
 humMRP CAGTGGACTCCATGATCCCGAGGTTCATCAAGATGTTTCATGGGCTCCCTG 3294  
 chMRP TTTAACGTGATTGGGGCTTGATCATCATTTTGTGCTGGCCACCCCTATAGC 841  
 || ||||| ||||| || ||||| || ||||| ||||| ||||| ||  
 humMRP TTCAACGTCAATTGGTGCCTGCATCGTTATCCTGCTGGCCACGCCCATCGC 3344  
 chMRP TGCTGTGCTTATTCCACCTCTGGGACTTGTCTACTTGTGCTGTGCAGAGAT 891  
 || | ||||| || ||||| || ||||| ||||| ||||| ||  
 humMRP CGCCATCATCATCCCGCCCTTGGCCATCTACTTCTTCGTCCAGAGGT 3394  
 chMRP TTTATGTGGCCACTTCTCGCCAGCTCAAACGCCCTTGAATCTGTTAGTCGT 941  
 | | |||| | || | ||||| || ||||| || ||||| ||  
 humMRP TCTACGTGGCTTCTCTCCGCGCAGCTGAAGCGCTCGAGTCGGTCAGCCGC 3444  
 chMRP TCTCCTGTGTATTCTCACTTCAATGAGACCCCTTCTGGGAGTCAGTGTAAT 991  
 || || || ||||| || ||||| ||||| ||||| ||||| ||  
 humMRP TCCCCGGTCTATTCCCATTTCAACGAGACCTTGTGTTGGGGTTCAGCGTCAT 3494  
 chMRP TCGAGCCTTTGAGGAACAGAAACGTTTATAAAGCAGAATGACATGAAAG 1041  
 ||||| ||||| ||||| || || ||||| ||||| ||||| ||  
 humMRP TCGAGCCTTCGAGGAGCAGGAGCGCTTCATCCACCAGAGTGACCTGAAGG 3544  
 chMRP TGGATGAAAATCAGAAAGCTTATTACCCAAGCATTGTTGCAAACAGATGG 1091  
 ||||| || ||||| ||||| ||||| ||||| ||||| ||||| ||  
 humMRP TGGACGAGAACCAGAAGGCCTATTACCCAGCATCGTGGCCAACAGGTGG 3594  
 chMRP CTGGCAGTACGTCTGGAGTTTGTGGGGAAGTATTGTTCTCTTTGCAGC 1141  
 ||||| || ||||| ||||| ||||| ||||| ||||| ||||| ||  
 humMRP CTGGCCGTGCGGCTGGAGTGTGTGGGCAACTGCATCGTTCTGTTTGTCTGC 3644  
 chMRP ATTGTTTGCAGTGATTGCACGCAACAAGCTCAGTCCGGGACTGATTGGTC 1191  
 ||||| ||||| | | || ||||| || ||||| ||  
 humMRP CCTGTTTGGCGTGATCTCCAGGCACAGCCTCAGTGTGGCTTGGTGGGCC 3694  
 chMRP TTTCAGTGTCTATTTCGCTGCAGATTACAGCATACTTAAACTGGCTAGTT 1241  
 | ||||| || ||||| || ||||| ||||| ||||| ||||| ||  
 humMRP TCTCAGTGTCTTACTCATTCAGGTCACCACGTACTTGAAGTGGCTGGTT 3744  
 chMRP CGTATGACATCTGATCTGGAAACCAACATTTGTTGCTGTAGAAAGAGTCAA 1291  
 || ||| ||||| ||||| ||||| || ||||| ||||| ||||| ||  
 humMRP CGGATGTCTATCTGAAATGGAAACCAACATCGTGGCCGTGGAGAGGCTCAA 3794  
 chMRP AGAATATGCTGAAATGGAGAAGGAGGTACAG..... 1322  
 || ||| || || ||||| || ||||| ||  
 humMRP GGAGTATTGAGAGACTGAGAAGGAGGCGCCCTGGCAAATCCAGGAGACAC 3844

FIGURE 1 (cont.)

	801				850
chMRP				RPRRPIL	VMTDGEISEM
huMRP	AHVKGKHFEN	VIGPKGMLKN	KTRILVTHSM	SYLPQVDVII	VMSGGKISEM
murMRP	AHVKGKHFEN	VVGEMGLLKN	KTRILVTHGI	SYLPQVDVII	VMSGGKISEM
	851				900
chMRP	GSYQELLKQD	GAFAEFLRTY	ANAEQSMESS	DAS....SPS	GKERKPVENG
huMRP	GSYQELLARD	GAFAEFLRTY	ASTEQEQDAE	ENGVTGVSGP	GKEAKQMENG
murMRP	GSYQELLDRD	GAFAEFLRTY	ANAEQDLASE	DD...SVSGS	GKESKPVENG
	901				950
chMRP	VLVNDAPGKL	MHRQLSNSST	YSRETGKSQH	QSSTAELQKP	LAEK.NSWKL
huMRP	MLVTDASAGKQ	LQRQLSSSSS	YSGDI..SRH	HNSTAELQKA	EAKKEETWKL
murMRP	MLVTDTVGKH	LQRHLSNSSL	HSGDT..SQQ	HSSIAELQKA	GA.KEETWKL
	951				1000
chMRP	TEADTAETGR	VKATVYWEYM	KAIGLYISFL	SVFLFMCNHI	ASLASNYWLS
huMRP	MEADKAQTGQ	VKLSVYWDYM	KAIGLFISFL	SIFLFMCNHV	SALASNYWLS
murMRP	MEADKAQTGQ	VQLSVYWNYM	KAIGLFITFL	SIFLFMCNHV	SALASNYWLS
	1001				1050
chMRP	LWTDD.PVVN	GTQQYTNVRL	GVYGALGISQ	GIAVEFGYSMA	VSIGGIFASR
huMRP	LWTDD.PIVN	GTQEHTKVRL	SVYGALGISQ	GIAVEFGYSMA	VSIGGILASR
murMRP	LWTDDPPVVN	GTQANRNFR	SVYGALGILQ	GAAIFGYSMA	VSIGGIFASR
	1051				1100
chMRP	HLHLDLLHNV	LRSEMSFFER	TPSGNLVNRF	SKEIDTIDST	IPPIIKMFMG
huMRP	CLHVDLLHSI	LRSEMSFFER	TPSGNLVNRF	SKELDTVDSM	IPEVIRMFMG
murMRP	RLHLDLLYNV	LRSEMSFFER	TPSGNLVNRF	SKELDTVDSM	IPQVIKMFMG
	1101				1150
chMRP	STFNVIGACI	IILLATPIAA	VVIPPLGLVY	LLVQRIYVAT	SRQLKRLESV
huMRP	SIFNVIGACI	VILLATPIAA	IIIPPLGLIY	FFVQRFYVAS	SRQLKRLESV
murMRP	SIFSVIGAVI	IILLATPIAA	VIIPPLGLVY	FFVQRFYVAS	SRQLKRLESV
	1151				1200
chMRP	SRSVPYSHFN	ETLLGVSVIR	AFEEQKRFIK	QNDMKVDENQ	KAYYPSIVAN
huMRP	SRSVPYSHFN	ETLLGVSVIR	AFEEQERFIH	QSDLKVDENQ	KAYYPSIVAN
murMRP	SRSVPYSHFN	ETLLGVSVIR	AFEEQERFIH	QSDLKVDENQ	KAYYPSIVAN
	1201				1250
chMRP	RWLAVRLEFV	GNCIVLFAAL	FAVIARNKLS	PGLIGLSVSY	SLQITAYLNV
huMRP	RWLAVRLECV	GNCIVLFAAL	FAVISRHSLS	AGLVGLSVSY	SLQVTTYLNV
murMRP	RWLAVRLECV	GNCIVLFAAL	FAVISRHSLS	AGLVGLSVSY	SLQITAYLNV
	1251				1300
chMRP	LVRMTSDLET	NIVAVERVKE	YAEMEKE		
huMRP	LVRMSSEMET	NIVAVERLKE	YSETEKEAPW	QIQETRPSS	WPQVGRVEFR
murMRP	LVRMSSEMET	NIVAVERLKE	YSETEKEAPW	QIQETAPPST	WPHSGRVEFR

FIGURE 2

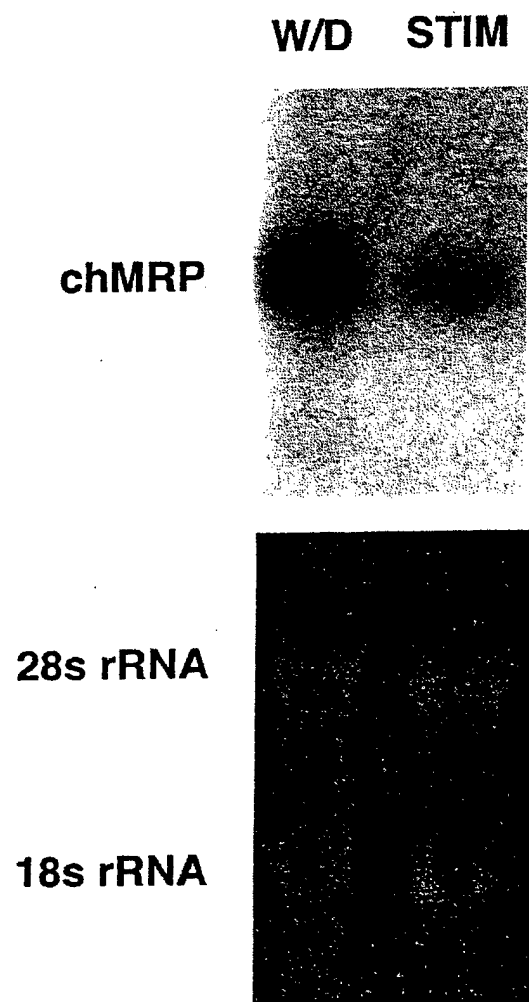


FIGURE 3



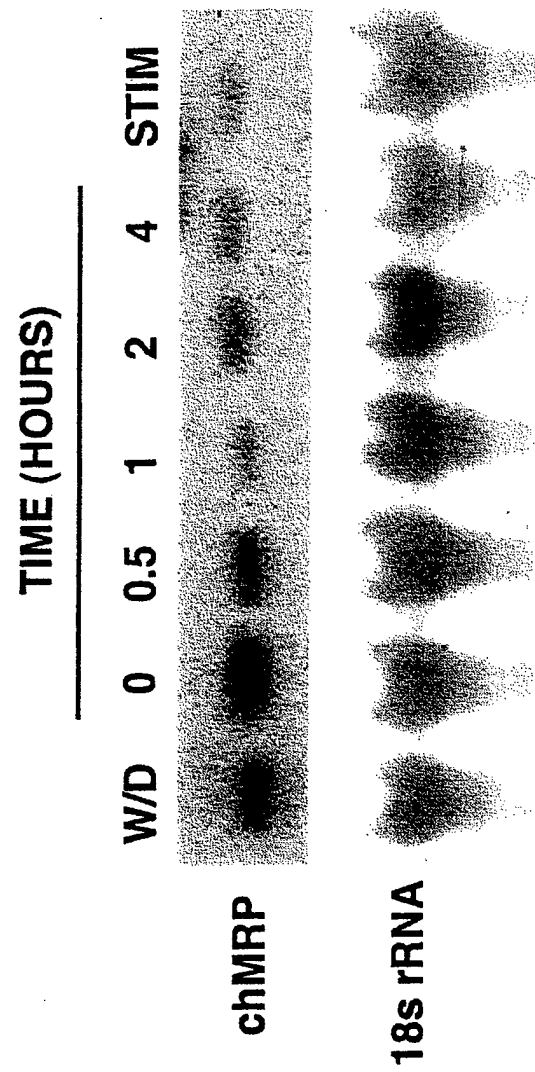


FIGURE 4A

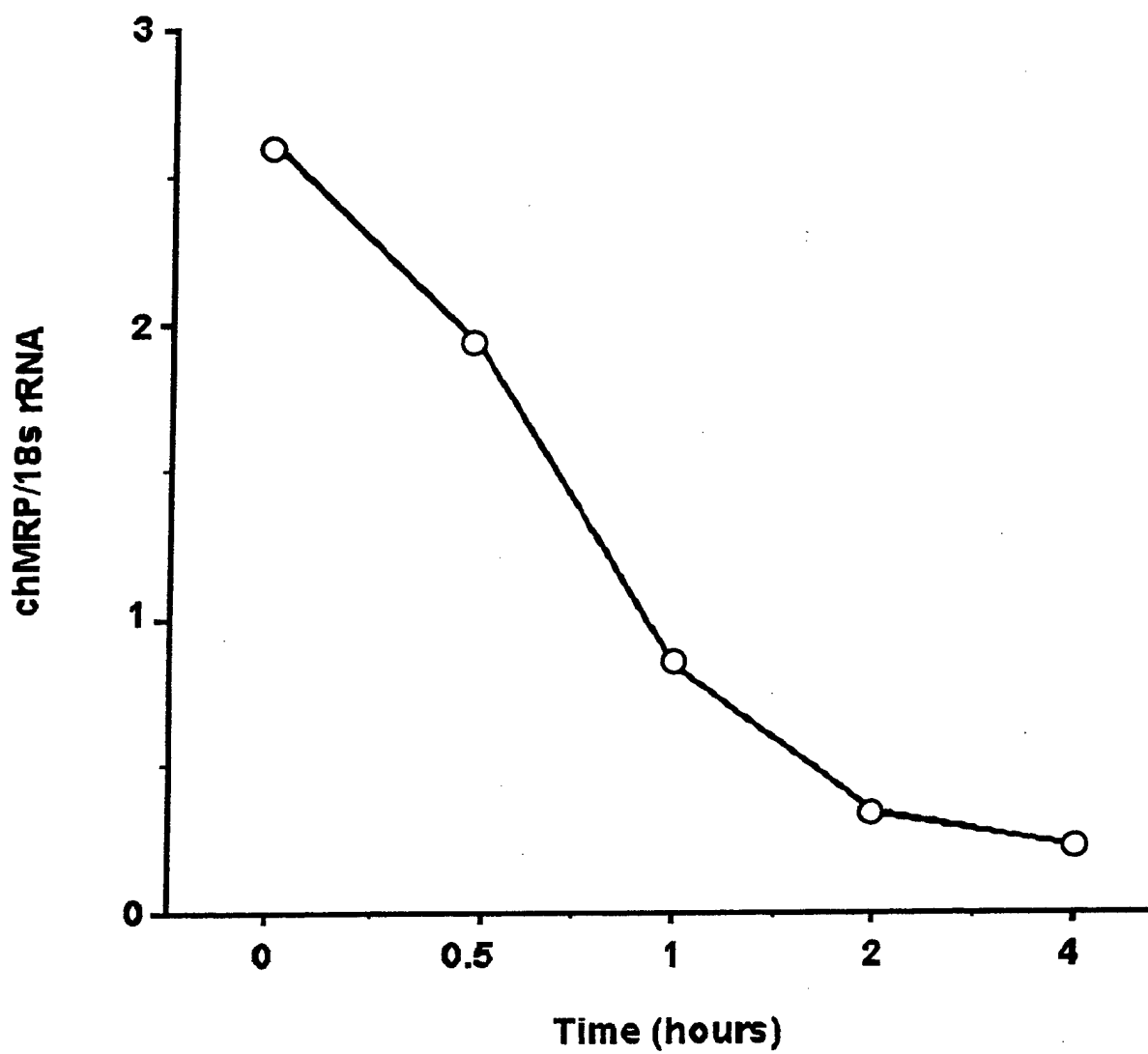


FIGURE 4B

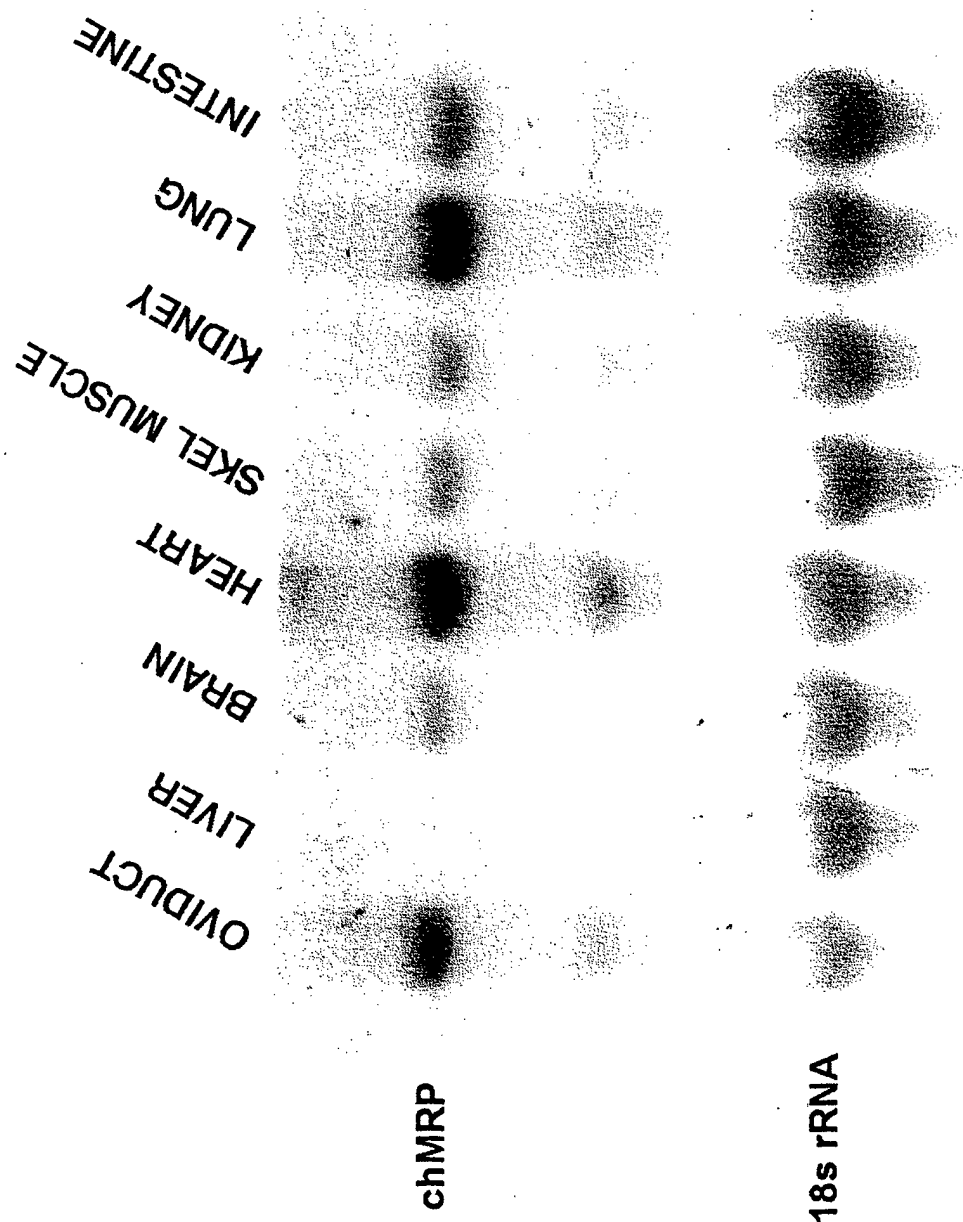
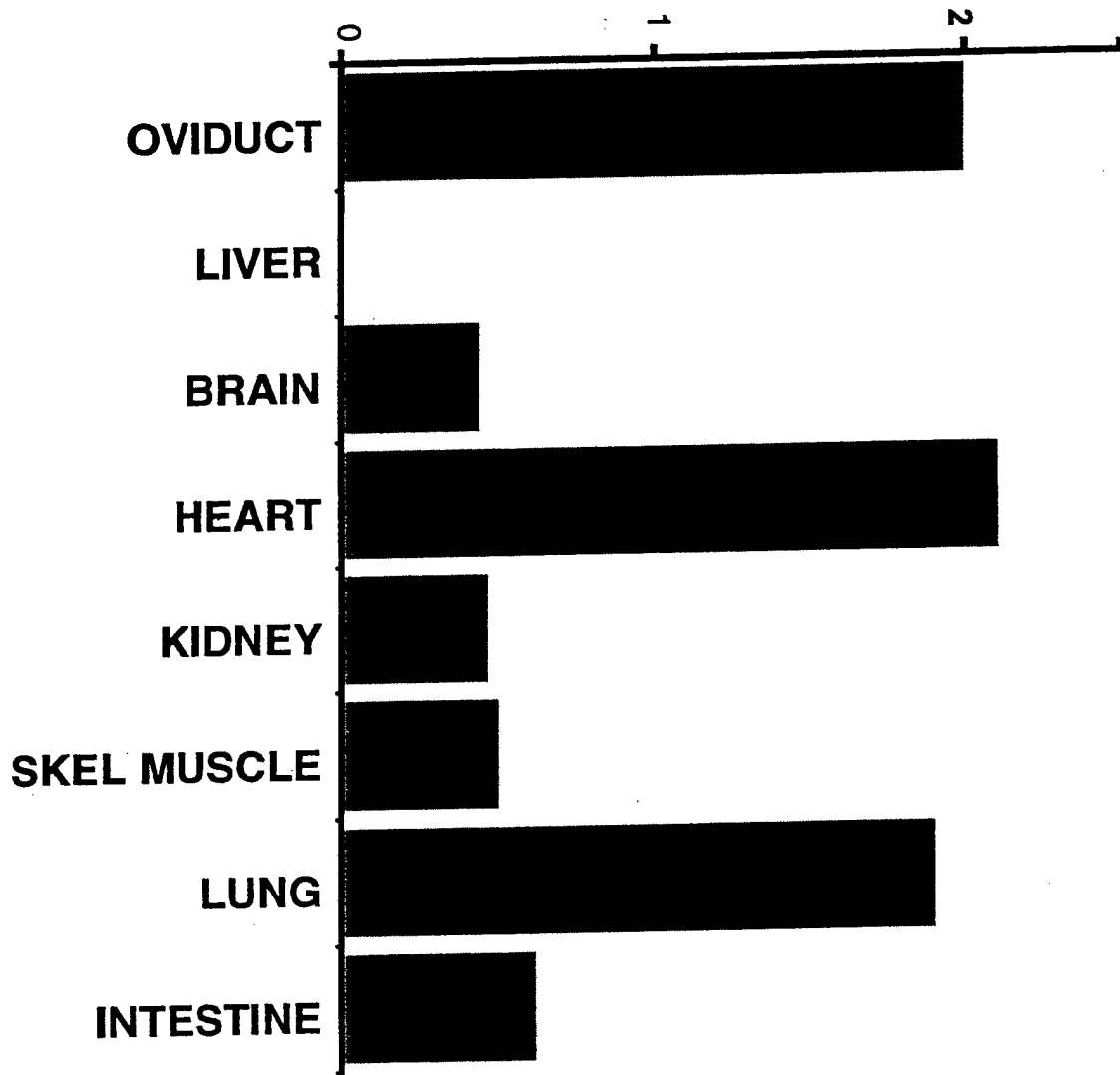


FIGURE 5A

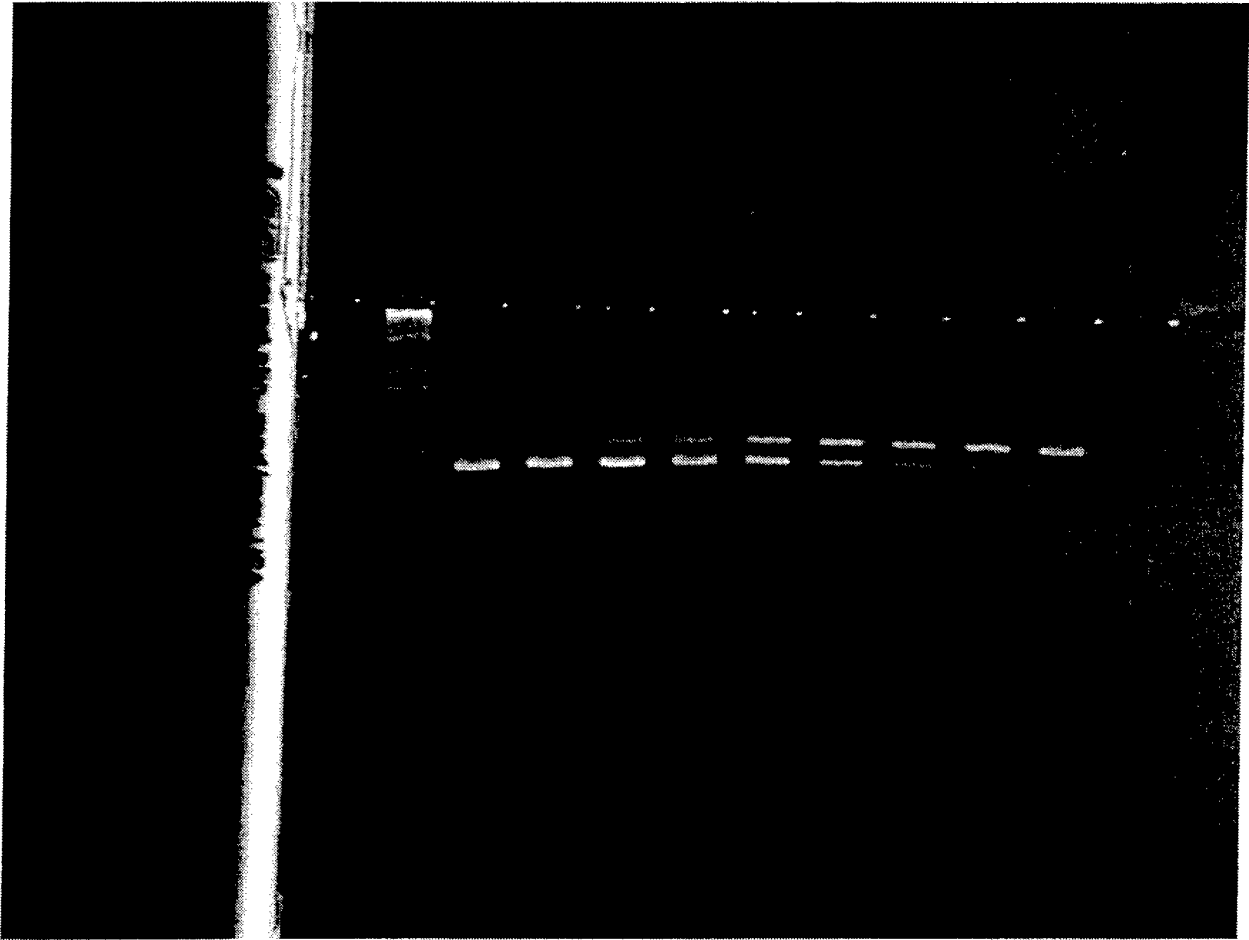
**chMRP/18s rRNA**



**FIGURE 5B**

**APPENDIX B**

- Figure 1:** Sample quantitative, competitive RT-PCR reaction
- Figure 2:** Sample quantitative, competitive RT-PCR reactions from MCF-7 cells treated with estrogen
- Figure 3:** Estrogen reduces the half-life of MRP mRNA to 2 hours in MCF-7 cells



**Figure 1 : Sample quantitative, competitive RT-PCR reaction.**

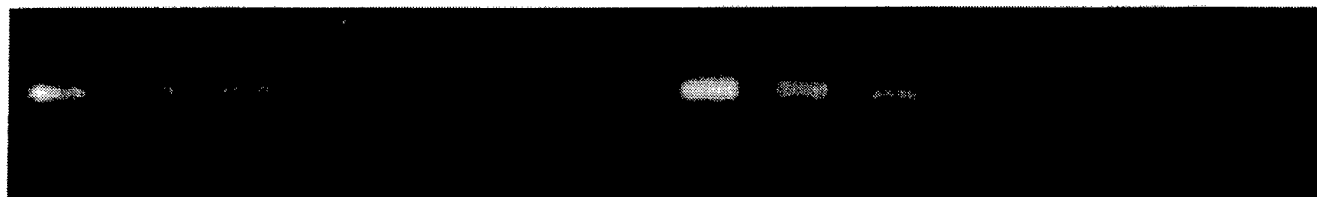
Total RNA was isolated from MCF-7 cells and subjected to quantitative, competitive RT-PCR using an internal control molecule. ICM was added in 2-fold decreasing concentrations from 400 fg to 3.125 fg (lower band). The upper band is the reaction achieved with the target molecule, human MRP mRNA, with a constant amount (2 ug) of the reverse transcription reaction. The amount of MRP mRNA in the sample is calculated using linear regression analysis of the ICM but basically represents the point where both bands are of equal intensity.

**Figure legend 2:** Sample quantitative, competitive RT-PCR reactions on MCF-7 cells treated with estrogen.

MCF-7 cells were cultured in charcoal stripped serum for 1 day prior to the experiment. At zero time,  $1 \times 10^7$  M estradiol was added. The cells were then harvested 1, 2, or 4 hours later, the RNA was isolated, and subjected to quantitative, competitive RT-PCR using an internal control molecule. ICM was added in 2-fold decreasing concentrations from 400 fg to 12.5 fg. Gels are shown for selected samples. The upper band is the reaction achieved with the target molecule, human MRP mRNA. The bottom band represents the internal control molecule. The amount of MRP mRNA in the sample is calculated using linear regression analysis of the ICM but basically represents the point where both bands are of equal intensity.

<u>Sample Number</u>	<u>Treatment</u>
1 – 4	0 time
5 – 8	0.5 hrs +E
9 – 12	0.5 hrs –E
13 – 16	1 hr +E
17 – 20	1 hr –E
21 – 24	2 hrs +E
25 – 28	2 hrs –E
29 – 32	4 hrs +E
32 – 36	4 hrs –E

## APPENDIX B, Figure 2



Gel 1a exp#232 1-22-99  
MCF7 time course samples 1 and 2



Gel 1b exp#232 1-22-99  
MCF7 time course samples 7 and 8



Gel 2a exp#232 1-22-99  
MCF7 time course samples 9 and 10



Gel 2b exp#232 1-22-99  
MCF7 time course samples 15 and 16



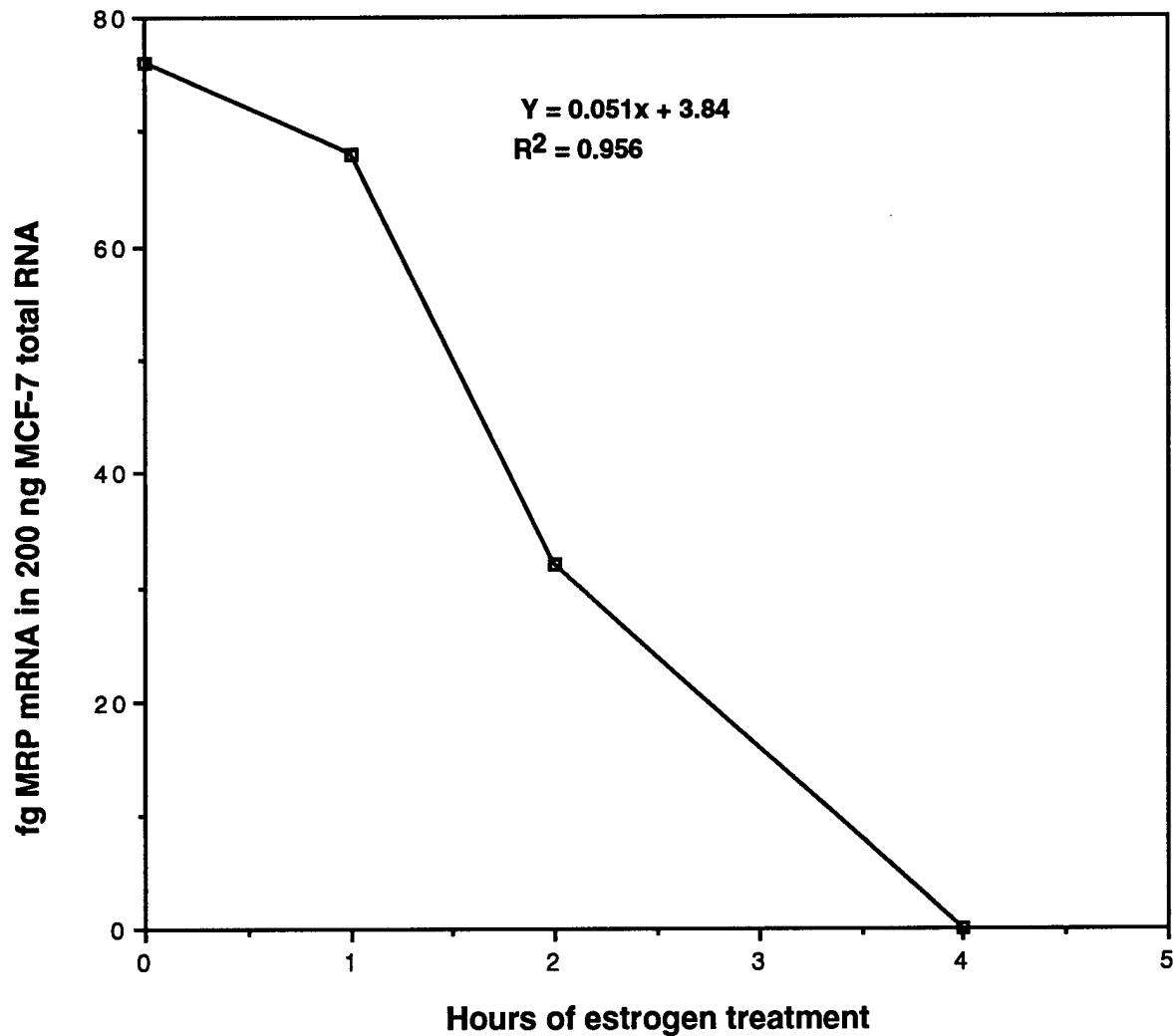
Gel 3a exp#232 1-22-99  
MCF7 time course samples 18 and 17



Gel 3b exp#232 1-22-99  
MCF7 time course samples 11 and 13



# Estrogen reduces the half-life of MRP mRNA to 2 hours in MCF-7 cells



**Figure 3: Estrogen reduces the half-life of MRP mRNA to 2 hours in MCF-7 Cells**

MCF-7 cells were cultured in charcoal stripped serum for 1 day prior to the experiment. At zero time,  $1 \times 10^7$  M estradiol was added. The cells were then harvested 1, 2, or 4 hours later, the RNA was isolated, and subjected to quantitative, competitive RT-PCR using an internal control molecule. The ethidium bromide stained gel was quantitated using software from Molecular Analysts.